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14. ABSTRACT:

This proposal is to design and evaluate a novel class of bifunctional MDM2 inhibitors, based on the discovery that nucleotides can bind to the P-loop of MDM2 and cause its relocalization to the nucleolus. Such bifunctional compounds will be designed to target MDM2, but not other P-loop-containing proteins. This approach represents a new strategy for the inhibition of MDM2 function and the treatment of breast cancer. During the first year of this grant we have (1) cloned and expressed GST-fused Mdm2 wild-type RING domain and its point mutations; (2) procured and tested all commercially available fluorescently labeled ATP analogs and identified 2 specific high affinity binders to the Mdm2 RING domain; (3) created a structure-activity relationship model (SAR) of the Mdm2 RING domain based on filter binding data of known ATP analogs for use in structure-based virtual screening; (4) developed high-throughput filters to exclude toxic and reactive compounds from commercial compound libraries, and (5) performed virtual screen on pre-filtered compound libraries and retrieved 800 candidate compounds for testing. Going forward we will build on these accomplishments to identify and optimize novel compounds that interact with and inhibit the E3 ligase activity of Mdm2.

15. SUBJECT TERMS
MDM2, p53, ring, ubiquitin ligase, combinatorial chemistry, high-throughput screening, chemical biology, chemical genetics, synthetic lethality

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Introduction

The Mdm2 protein plays essential roles in negatively regulating the p53 tumor suppressor protein. Mdm2 has been found to be upregulated in a significant number of breast cancers. The Mdm2 RING domain possesses a cryptic nucleolar localization signal sequence and a nucleotide-binding P-loop motif. These two motifs are unique to the Mdm2 RING and are not present in the myriad other RING domains encoded in the human genome. Nucleolar relocalization of Mdm2 causes p53 stabilization and activation, and nucleotide binding to the Mdm2 RING domain facilitates Mdm2 nucleolar localization.

Mdm2 has been found to play an essential role in negatively regulating the p53. In turn, the p53 protein binds to the promoter of Mdm2 and activates Mdm2 transcription. The importance of Mdm2 in p53 down-regulation has also been shown by genetic analysis: loss of *mdm2* in mice results in early embryonic lethality, but deletion of *p53* with *mdm2* rescues the lethal phenotype.

There is mounting evidence for a pro-oncogenic role for Mdm2 that is independent of p53 in both murine models and in human tumor cohort studies. Therefore the novel inhibitors that we plan to develop may have broader impact than activation of p53.

The design of genetically-targeted anti-tumor agents is an important new strategy in cancer drug discovery. However, it is often difficult to identify the specific proteins that should be targeted for maximal clinical benefit and to develop small molecules that target these proteins. We have identified an opportunity to develop a potentially powerful new class of compounds targeted against breast cancers with a specific genetic modification. We propose to develop genetically targeted small molecules that will selectively eradicate breast cancer cells containing amplified or up-regulated Mdm2.

Project Progress - Body

We have cloned, bacterially expressed, and affinity purified GST-fused Mdm2 wild-type RING domain as well as several alanine point mutations, including the previously published Mdm2 K545A P-loop mutation that exhibits decreased ATP binding affinity (Poyurovsky et al., 2003). We have assayed these proteins for ATP binding and specificity using an *in vitro* filter binding assay. Mdm2 proteins were also tested for E3 ligase activity using an *in vitro* ubiquitin polymerization assay. We have identified a C-terminally deleted Mdm2 RING domain protein that does not oligomerize in solution and exhibits dramatically increased solubility when compared to similarly expressed and purified wild-type Mdm2 RING (Figure 1). Although this protein is deficient in E3 ligase activity, it binds ATP similarly to wild type Mdm2 and coordinates a stoichiometric quantity of zinc, suggesting that it is properly folded. This protein is ideal for development of a high-throughput assay due to its high bacterial yield and excellent solubility.

Using this protein, we have performed *in vitro* filter binding assays to test all commercially available fluorescently labeled ATP analogs. We have identified two of these analogs that bind to the Mdm2 RING domain with low micromolar affinity, as well or better than unlabeled ATP (Figure 2). These analogs will be used in a high-throughput fluorescence polarization assay to test candidate compounds for binding to the Mdm2 RING domain.

We have also made progress optimizing a high-throughput fluorescence polarization assay using these fluorescently labeled ATP analogs. We are currently in the process of calibrating the concentrations of protein and fluorescently labeled analog in order to obtain and reliable and interpretable signal.

As compound toxicity and scaffold suitability are major concerns in any inhibitor development, we used an *in silico* filtering protocol to select such compounds for inclusion in the high-throughput screen from among 1,322,183 candidate compounds available from five reliable chemical suppliers. The selection criteria included filters for excluding reactive molecules and those with unsuitable scaffolds, unsuitable referring to compounds with obvious side-effects and not drug-like scaffolds. The filtering scripts were written in SVL and used the MOE 2004.03 software suite(CCG, 2004). The filters were based on the previous work of Hann et al. (Hann et al., 1999.) and used a SMILES-string-based search method (Weininger et al. 1998.). SMILES strings are a simple, text-based way of representing molecules, allowing for faster structure-based searches while being as reliable as computationally more expensive methods. From the original 1.3 million compounds, 485120 compounds were selected.

These compounds were then subjected to pharmacophore-based virtual screening to select for compounds similar to ATP. The model for this screen was designed by aligning several known ATP and purine nucleotide analogs in order to find their common structural features. These analogs were then tested for MDM-RING-domain binding and their affinity determined. Based on the analogs' binding affinity and the superposition of their structure, a Quantitative Structure-Activity Relationship model was built.(Figure 3.) This model served as the basis for the pharmacophore model , which included hydrogen-bond acceptors for the ribose-ring's 2' and 3' hydroxyls, a hydrogen-bond donor for the 6'

amine group on the purine ring and an anionic group of the beta-phosphate(Figure 4.). The screen was conducted using MOE on previously filtered libraries. For the screen, all compounds were pre-processed by assigning polar hydrogen atoms and charges according to the MMFFx forcefield (Halgren et al., 1996.), any salts were converted to free-base or free-acid form. Conformer libraries for the screen were generated using OMEGA 1.8, set to 200 conformers per molecule. In total, the screen yielded 2000 hits.

For each hit compound, one conformer with the lowest root-mean-square deviance was selected. The library of hit compounds was then submitted to clustering to remove close structural homologs frequently created in combinatorial synthesis. This was achieved by annotating the compounds with MACCS (MDL Corporation) structural keys; the clustering itself was performed using the Jarvis-Patrick method. Molecular similarity was determined by the Tanimoto coefficient, the threshold for which was set at 0.7. Of the resulting 800 compounds, 150 have been tested up to date. Other pharmacophore models with different structural constraints have also been developed to retrieve other adenine-like hits.

Key Research Accomplishments

- Cloned and expressed GST-fused Mdm2 wild-type RING domain and its point mutations.

- Procured and tested all commercially available fluorescently labeled ATP analogs and identified 2 specific high affinity binders to the Mdm2 RING domain.

- Created a structure-activity relationship model (SAR) of the Mdm2 RING domain based on filter binding data of known ATP analogs for use in structure-based virtual screening.

- Developed high-throughput filters to exclude toxic and reactive compounds from commercial compound libraries.

- Performed virtual screen on pre-filtered compound libraries and retrieved 800 candidate compounds for testing.

Reportable Outcomes

Posters

13th p53 Workshop, May 20-24, 2006. Columbia University, New York, NY, USA.

Deconstructing Nucleotide Binding Activity of the Mdm2 RING Domain

Christina Priest, Masha Poyurovsky, Brent Stockwell and Carol Prives
Department of Biological Sciences, Columbia University, New York, NY 10027

The RING domain of Mdm2 contains a conserved Walker A or P-loop motif characteristic of nucleotide binding proteins. As has been previously shown, Mdm2 preferentially binds adenine base nucleotides and that such binding leads to a conformational change in the Mdm2 C-terminus. (Poyurovsky et al. Mol Cell. 12: 875-87, 2003). Nucleotide binding defective Mdm2 mutants are impaired in p14/ARF-independent nucleolar localization both *in vivo* and *in vitro,* and ATP-bound Mdm2 is preferentially localized to the nucleolus.

Here we present further biochemical analysis of the nucleotide-Mdm2 interaction. We confirmed the original ATP binding and specificity results using Isothermal Titration Calorimetry (ITC). Further investigation of the interaction using a series of ATP derivatives identified 2' and 3' hydroxyls of the ribose as well as the C6 amino group of the adenine base moiety as being essential for the interaction. These results further support our previous data on ATP specificity, as the C6 amino group is a unique feature of adenine. MdmX, an Mdm2 family protein with high sequence homology, similarly binds adenine nucleotides preferentially. In order to further elucidate the structural features of Mdm2 necessary for ATP interaction, we have created a series of substitution mutations in residues within the Mdm2 RING domain that were predicted to be involved in base recognition. We assayed the resulting mutant proteins for nucleotide binding, nucleotide specificity, and E3 ligase activity. Our results highlight an intriguing separability between nucleotide binding and E3 functions of the Mdm2 RING domain, indicating that this domain may be involved in several unrelated biochemical processes. (Supported by DOD propoposal #BC044468)

Conference presentations:

10th Annual Spinal Muscular Atrophy Research Group Meeting
June 10-12th, Montreal, Canada

High-Throughput Screen for Compounds that Increase SMN Protein Level

Reka Letso, Columbia University, Laboratory of Dr. Brent Stockwell

Spinal muscular atrophy (SMA) is a pediatric neurodegenerative disease in which nearly all patients have a homozygous deletion of the *survival of motor neuron 1 (smn1)* gene. However, patients retain at least one copy of the nearly identical gene *smn2,* which

cannot fully compensate for the deleted gene due to increased use of an alternative splice site. Due to the existence of this modifiable gene isoform, SMA is an exceptional candidate for small molecule intervention. Therefore, we have undertaken a high-throughput screen in patient fibroblast cells to discover small molecules which increase endogenous SMN protein levels. To date we have screened ~30,000 compounds from our unique chemical libraries which were chosen using the latest cheminformatic approaches for selection of drug-like compounds with increased likelihood of crossing the blood-brain barrier. One hit has been confirmed and further characterization of its mechanism of action may help elucidate the molecular basis of SMA.

Conclusions

In summary, we made good progress on the tasks we laid out one year ago. In the upcoming years, we will focus on finalizing the assays we will use to test large numbers of compounds. In addition, we will optimize the structures of MDM2 ligands to improve potency and selectivity. These studies may lead to the discovery novel, selective MDM2 inhibitors that are effective treatments for breast cancers.

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Molecular Design Limited Corporation.

Poyurovsky, M. V., Jacq, X., Ma, C., Karni-Schmidt, O., Parker, P. J., Chalfie, M., Manley, J. L., and Prives, C. (2003). Nucleotide binding by the Mdm2 RING domain facilitates Arf-independent Mdm2 nucleolar localization. Mol Cell *12*, 875-887.

Weininger, D. (1998) SMILES 1. Introduction and Encoding Rules , J.Chem. Inf. Comput. Sci. 28-31

Appendix/Supporting Data

(a) (b)

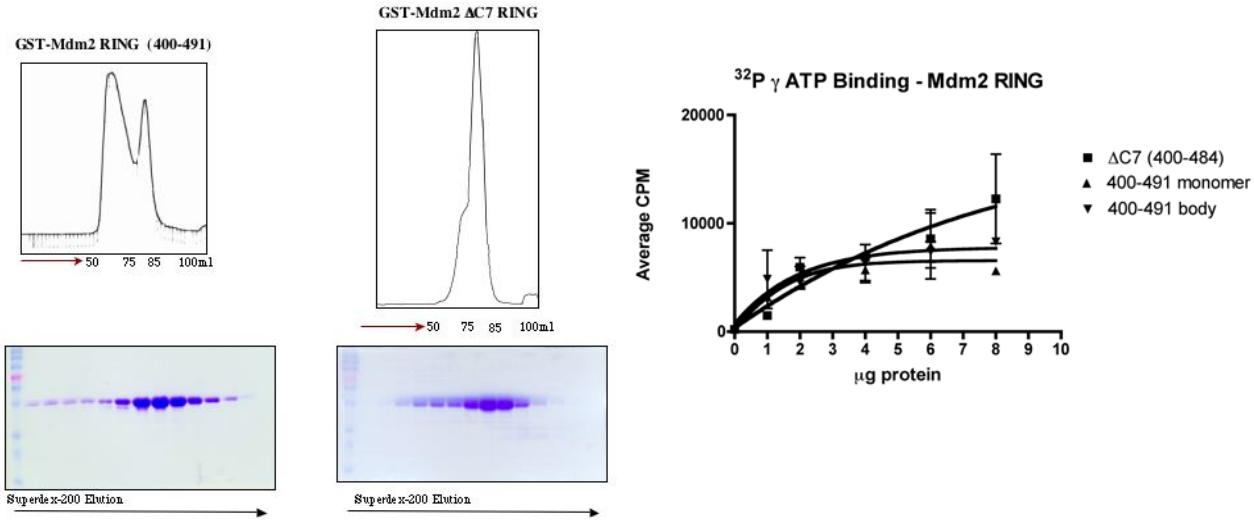
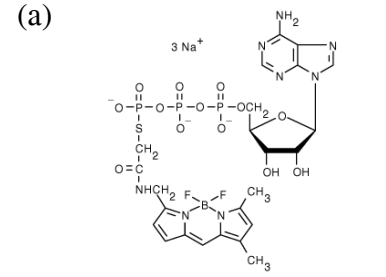


Figure 1
Gel filtration profile and (b) ATP binding comparison of full-length GST-Mdm2 RING construct (residues 400-491) and ΔC7 GST-Mdm2 RING construct (residues 400-484).

(a) (b)



Compound	K_d (μM)	Std. Err.
BODIPY FL ATP-γ-S thioether	0.7779	0.08318
Fluorescein-12-ATP	0.142	0.03782

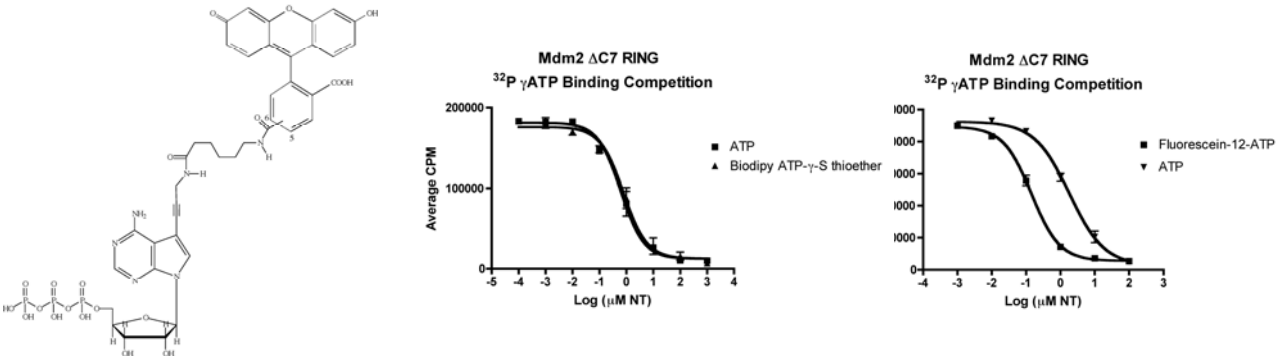


Figure 2
(a) Fluorescently labeled ATP analogs that bind to Mdm2 RING domain with similar or better affinity as unlabeled ATP (b) Disassociation constants and binding competition curves of fluorescent ATP analogs.

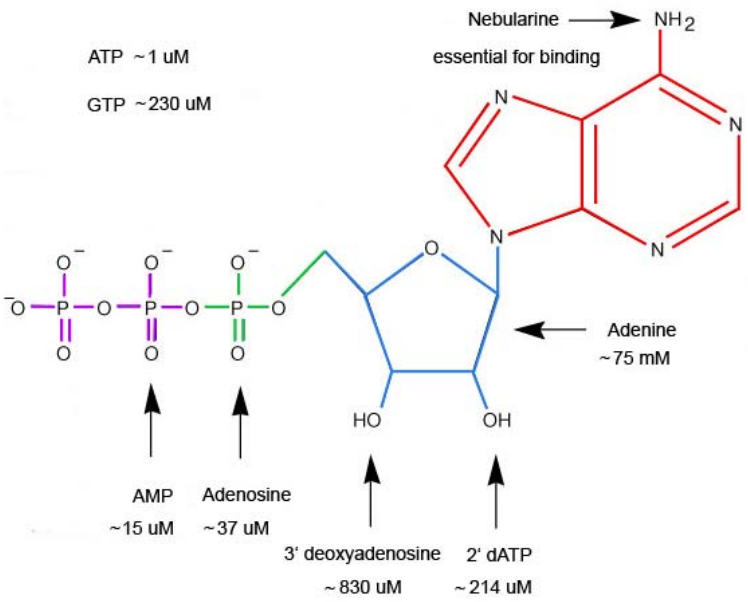


Figure 3
Features of ATP required for Mdm2 binding as indicated by disassociation constants (K_d) of ATP derivatives.

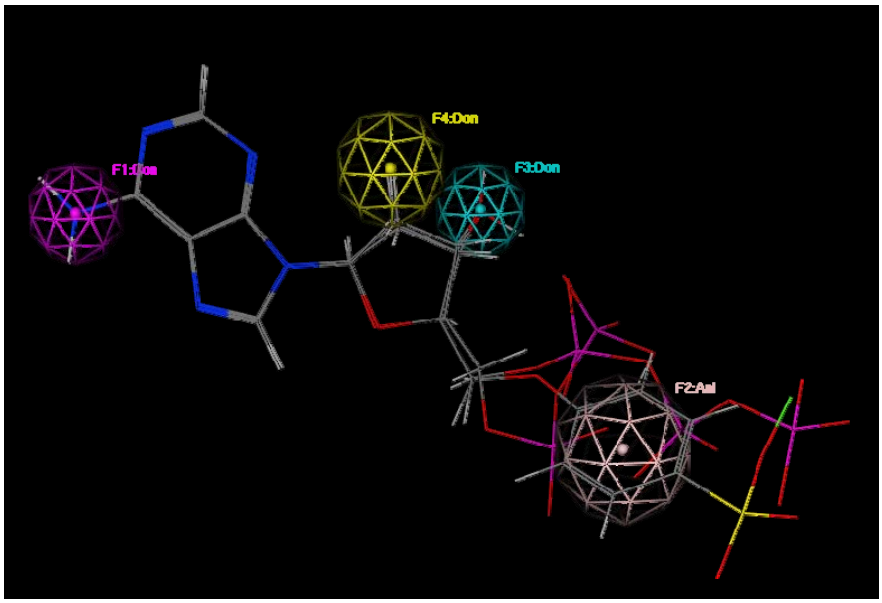


Figure 4.
Pharmacophore model used for ligand-based virtual screening, superposed with ATP and ATP analogs ADP and FSBA . F1: Hydrogen-bond donor, F2,3: Hydrogen-bond acceptors, F4: Anionic group